

# SINGLE PHOTOMULTIPLIER TECHNOLOGY FOR SCINTILLATION COUNTING IN MICROPLATES

*BERNIE EFFERTZ, KEN NEUMANN and DAVID ENGLERT*

Packard Instrument Company, 1 State Street, Meriden, Connecticut 06450 USA

**ABSTRACT.** Counting radioisotopes in microplates requires special technology to count with good efficiency and negligible crosstalk between wells. In the TopCount™ Microplate Scintillation Counter, we use time-resolved counting technology to achieve high counting efficiencies and low backgrounds with a single photomultiplier tube (PMT), which collects light from the top of the microplate well. We developed liquid and solid scintillants that produce slowly decaying pulse trains, and the electronics to discriminate true beta disintegration events from noise based on the pulse characteristics. Crosstalk is prevented by the basic design of the instrument and the use of opaque, reflective microplates. Crosstalk from <sup>3</sup>H or <sup>14</sup>C is not measurable above background, whereas crosstalk from penetrating radiation, such as <sup>125</sup>I or <sup>32</sup>P, is measurable, but only a fraction of a percent. With the virtual absence of crosstalk, sample spectra are not corrupted by small crosstalk pulses, and the sample spectra (tSIS) can be used for reliable quench correction. With 24-well microplates, it is possible to introduce an external standard for quench correction using the external standard spectrum (tSIE), and accurate dual-label counting can be performed. Fast, single-photon detection circuitry permits luminescence measurements using two detectors with a dynamic range of up to 10<sup>6</sup> and with crosstalk of less than 0.03%.

## INTRODUCTION

Microplates have become very popular in biological research, especially for high-throughput assays. Microplates permit high sample densities and reductions in sample volume, reagent volume, labor, and chemical and radioactive waste. The TopCount™ Microplate Scintillation Counter was designed so that a variety of assays could be performed in microplates with either radioisotope or luminescent labels without compromising the reliability of the results.

A fundamental design consideration was that microplates should be opaque to prevent light from traveling between the closely spaced wells. The instrument was designed for efficient counting of samples deposited on the surfaces of opaque or translucent media, such as solid scintillators, filters and membranes. It was important that the instrument be compatible with a variety of standard microplate formats, which could be used with standard microplate sample preparation instrumentation, including robotic liquid handling systems. These considerations led to the single photomultiplier tube (PMT) design of TopCount™. With this design, each of up to 12 PMTs detects photons from the top of individual microplate wells.

We describe here the basic design of TopCount™ and present results that illustrate its performance for quantitation of both radioactive and luminescent labels. The success of this design in preventing crosstalk among wells permits accurate quantitation with good dynamic range, even with closely spaced samples that have large differences in activity and variable quench. The design provides good counting efficiency with a variety of formats, including heterogeneous, non-transparent media.

## TOPCOUNT™ DESIGN

The counting chamber of TopCount™ consists of the opaque microplate well, the PMT and a collimator between the microplate and PMT (Fig. 1). The microplate and the collimator are made of highly reflective white material, so that the photons from the sample are efficiently collected by the PMT. The collimator is designed to precisely position the PMT, and to prevent the escape of light to other detectors. The dense colorants in the plates also absorb the energy of penetrating radiation to minimize crosstalk from high-energy radionuclides.

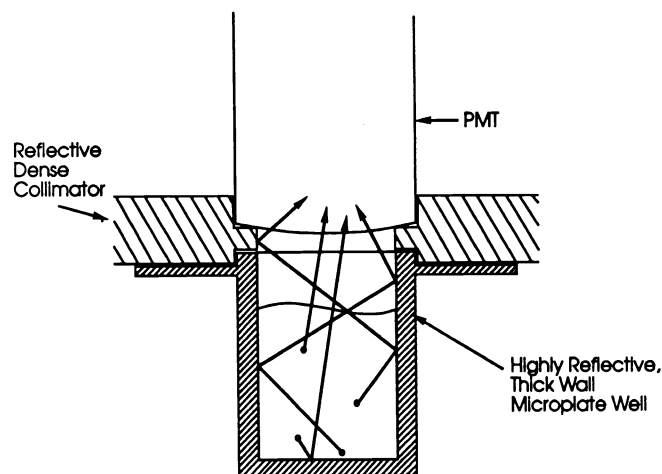


Fig. 1. Single PMT design of TopCount™. Light from scintillations or luminescence is reflected from the opaque microplate walls to the PMT. A collimator between the microplate and PMT prevents light leakage to other wells and prevents contamination of the PMT.

Several manufacturers offer compatible microplates to perform assays involving luminescence, standard liquid or solid scintillation counting (LSC, SSC), filtration, binding to membrane or plastic surfaces, cell culture, or scintillation proximity assay (SPA). Plates in 96- or 24-well formats may be counted. The spacing between wells in the 24-well format is sufficient to permit the introduction of a  $^{133}\text{Ba}$  gamma source to produce a spectrum of Compton electrons for external standardization.

To discriminate electronic pulses produced by scintillation events from PMT noise, conventional scintillation counters accept only events that cause coincident pulses (within a few nanoseconds) in two PMTs. Time-Resolved Liquid Scintillation Counting (TR-LSC™) in TopCount™ can discriminate scintillation events from noise. Photon emission from scintillation events is delayed compared to conventional LSC, so that multiple electronic pulses from single decay events can be detected within a time window of 200 nsec (Fig. 2). The circuitry accepts only events that produce 2–3 pulses larger than single photoelectron (SPE) pulses within the time window.

The MicroScint™ series of LS cocktails store part of the energy from radioactive disintegrations for 15 nsec or longer. The relatively slow decay of this energy results in multiple electronic pulses for each radioactive decay in the single PMT (Fig. 2). The solid scintillator, yttrium silicate, produces delayed scintillation events that can be counted by TR-LSC™. This scintillator has been incorporated into LumaPlates™, in which liquid samples can be dried for scintillation counting. The beads used in SPA kits (Amersham International plc) also produce delayed scintillations.

Specialized single photon counting (SPC) circuitry was designed for quantitation of luminescence. The pulse pair resolution time of these ultra-fast circuits is 11 ns, permitting quantitative photon flux measurements of  $>10^7 \text{ sec}^{-1}$ . Because the PMT background is *ca.*  $3 \times 10^2 \text{ sec}^{-1}$ , the dynamic range is  $\sim 10^6$ . The photodetectors in TopCount™ are maintained below ambient temperature to reduce the electronic noise. This temperature is regulated to within 1°C to ensure stable and uniform counting efficiencies for all photomultipliers, independent of the ambient temperature.

## METHODS

We performed experiments on a TopCount™ equipped with the VariPlate™ feature, which permits counting in either 24- or 96-well microplates. Dilutions of food coloring or nitromethane were added to MicroScint™ scintillation cocktail to produce color or chemical quench, respectively. After

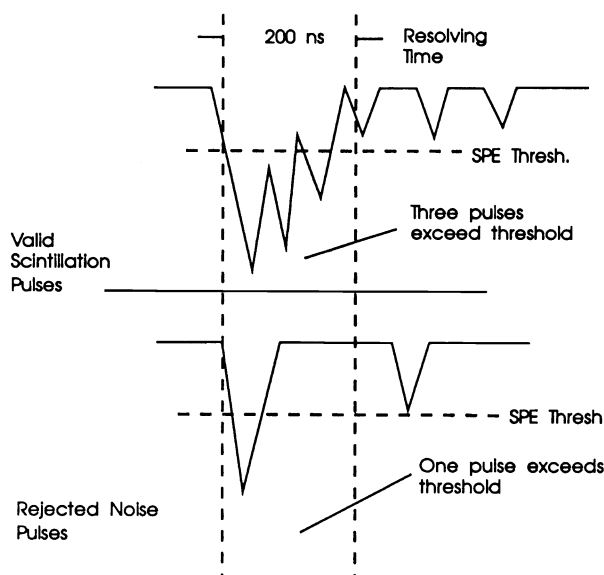


Fig. 2. Time-resolved single PMT background discrimination. Radioactive decays in a slow scintillator produce multiple pulses within a 200-ns period. Pulses larger than the single photoelectron (SPE) threshold are counted within this time window.

adding MicroScint™ cocktail when appropriate, plates were sealed with TopSeal™ film and counted using standard conditions. Reference measurements were made with a Tri-Carb® 2500TR LS counter or Cobra™  $\gamma$  counter.

To analyze well-to-well crosstalk, we prepared two dilutions of radionuclide with an activity difference of  $10^2$ . Ten-microliter aliquots of these dilutions were placed in selected wells of a microplate to measure crosstalk to surrounding wells, and to simulate experimental cases where relatively inactive samples are surrounded by active samples (Fig. 3). We also analyzed crosstalk from luminescent samples in a similar manner by preparing dilutions of xanthine oxidase and measuring chemiluminescence by the method of Baret *et al.* (1990). Wells containing no radioactivity or enzyme contained only scintillation fluid or the chemiluminescent signal reagent, respectively.

The average individual well crosstalk from a high-activity well (C3 in Fig. 3) into the surrounding eight wells is defined as

$$\frac{(\text{Average CPM of 8 wells around C3} - \text{Average CPM of surrounding 16 wells})}{\text{CPM of C3}} \times 100 \quad (1)$$

We made chemiluminescent determinations of alkaline phosphatase with Lumi-Phos® 530 (Lumigen®, Detroit, Michigan) or with AMPPD® (Tropix, Bedford, Massachusetts). A dioxetane substrate from Tropix was used to determine beta-galactosidase. The ECL reagent (Amersham, Arlington Heights, Illinois) was used to assay horseradish peroxidase. Luciferase was assayed with the Luciferase Reporter Gene Assay System (Promega, Madison, Wisconsin). Xanthine oxidase was determined with reagents kindly provided by Dr. Alain Baret (Laboratoire Trichereau, Nantes, France).

## RESULTS AND DISCUSSION

Table 1 lists counting efficiencies and figures of merit ( $E^2/B$ ) for both LSC and SSC in 24- and 96-well plates. Counting efficiencies for some other sample formats, such as filters, are discussed in this volume (Neumann *et al.* 1993). Solid or semi-transparent samples, such as the solid scintil-

A. C-14 Crosstalk in Polystyrene Plate (MicroLite)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	19	16	16	12	19	16	15	12	24	17	16	11
B	14	17	11	11	24	16	981	17	1030	40	1194	12
C	22	14	95188	6	12	18	14	10531	15593	106707	26	16
D	14	13	11	7	19	15	911	97102	1001	100640	1654	13
E	15	11	8	8	14	15	146	100331	105310	100979	18	14
F	16	16	1007	9	15	16	1302	11	1004	19	1050	14
G	19	17	8	13	24	16	13	11	22	22	20	12
H	16	12	11	11	40	19	18	12	15	17	11	13
B. 125I Crosstalk in Filter Bottom Plate (UniFilter)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	13	26	21	17	29	22	28	43	55	50	33	25
B	20	39	129	15	39	40	425	164	610	184	447	39
C	13	72	40487	112	29	42	139	37855	40313	41151	134	38
D	29	31	132	18	39	45	546	36746	890	39659	556	48
E	15	19	11	9	35	39	137	39206	40217	40694	114	42
F	9	19	382	8	22	39	381	152	585	130	401	36
G	17	19	25	21	19	24	35	44	55	40	29	35
H	15	18	15	22	18	16	17	22	23	28	21	24
C. XO Chemiluminescence Crosstalk in Black Plate (MicroFLUOR)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	213	213	245	225	206	148	113	153	173	120	207	60
B	173	270	832	213	161	173	31855	1847	54398	1747	46753	233
C	173	599	2824662	387	206	219	1440	3536639	3338967	3620460	633	93
D	161	232	483	283	199	199	59346	3412323	55867	3632547	43788	100
E	296	238	219	245	199	213	1500	3445719	3708672	3505933	567	93
F	303	206	59883	206	277	264	59553	713	48435	533	42460	120
G	528	187	219	251	1373	283	173	200	193	207	160	113
H	180	283	219	238	232	238	153	153	173	1013	507	147

Fig. 3. Evaluation of crosstalk with scintillation and luminescence counting. Uncorrected CPM results from TopCount™ are presented in a spreadsheet. Only wells in bold type contained radioactivity or enzyme activity. Shaded wells contained 100 times more activity than the unshaded wells. Outlined wells indicate wells that were used to calculate the amount of crosstalk (see text). A. No significant <sup>14</sup>C crosstalk above background in a white polystyrene plate (MicroLite™, Dynatech, Chantilly, Virginia). B. Slight <sup>125</sup>I crosstalk in a glass fiber filter bottom plate (UniFilter™, Packard, Meriden, Connecticut). C. Very slight xanthine oxidase chemiluminescence crosstalk under SPC conditions in a black polystyrene plate (MicroFLUOR®, Dynatech).

lator in LumaPlates™ or the filters in UniFilter™ plates, are counted with consistently high efficiency due to optimal counting geometry. The sample is positioned in a consistent orientation very close to the PMT, unlike materials placed in a vial for conventional LSC (Beckman Instruments 1989). With single PMT TR-LSC™, the relation between counting efficiency and the transformed spectral index of the sample (tSIS) or the transformed index of the external standard (tSIE) (Jones *et al.* 1986) is the same, regardless of whether the variable efficiency is due to chemical quench or color quench, and regardless of the color (Fig. 4A). Thus, a single quench curve can be used to correct for both forms and all colors of quench (Fig. 4B).

Experiments performed on TopCount™ with clear polystyrene plates have shown that the spectrum of optical crosstalk from neighboring wells is similar to the spectrum of a quenched sample (data not shown). Thus, it is not possible to distinguish crosstalk from quench on the basis of the sample spectrum, and reliable quench correction can be performed on TopCount™ only with opaque white plates that prevent optical crosstalk between wells. The single PMT design of TopCount™ permits the introduction of a <sup>133</sup>Ba source below the wells of a 24-well plate, so that the level of quench can be determined for DPM determination in dual-label experiments. DPM recovery errors are <10% for <sup>3</sup>H/<sup>14</sup>C activity ratios of 5:1 to 1:5 and a range of quench levels producing <sup>3</sup>H efficien-

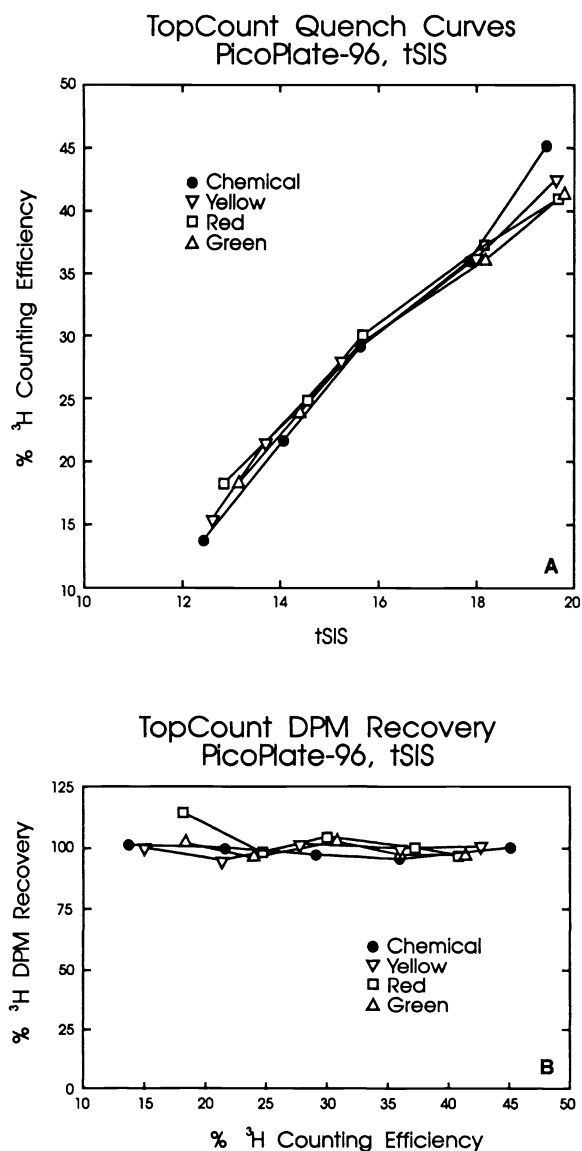


Fig. 4. Color and chemical quench correction with a single quench curve. A. Quench curve relating  $^3\text{H}$  counting efficiency and the tSIS for chemical quench produced by nitromethane ( $\bullet$ ) and for color quench produced by red ( $\square$ ), green ( $\triangle$ ), and yellow ( $\nabla$ ) dyes. B. DPM determination using the chemical quench curve in A for samples quenched to varying degrees with nitromethane and with colored dyes (symbols as in A).

cies of 10–50%. The energy of the  $\beta$  particles from low-energy isotopes, such as  $^{14}\text{C}$ , is too low to penetrate the material between wells; only optical crosstalk is a possible contributor. The  $^{14}\text{C}$  experiment in Figure 3A illustrates that optical crosstalk is virtually undetectable in TopCount™, even when a low-activity well (e.g., D9 in Fig. 3A) is surrounded by wells containing 100 times more activity. We have obtained similar results with  $^3\text{H}$  (data not shown).

Radiation from higher-energy radionuclides, such as  $^{125}\text{I}$ , can penetrate the material between wells to cause crosstalk by exciting the scintillant in neighboring wells. This is illustrated in Figure 3B, which shows slight crosstalk between wells of a 96-well filter bottom plate (UniFilter™ with glass fibers). Individual well crosstalk was 0.11%. The cumulative crosstalk into a low-activity well (e.g., D9) surrounded by wells with 100 times more activity can be seen in the right side of Figure 3B

TABLE 1. Efficiencies and Figures of Merit for Liquid and Solid Scintillation Counting of Isotopes Used in Biological Research

Isotope	% Efficiency (E)		E <sup>2</sup> /B	
	24-well	96-well	24-well	96-well
Liquid, <sup>3</sup> H	45	38	36	80
Liquid, <sup>14</sup> C	93	92	152	471
Liquid, <sup>125</sup> I	63	58	69	186
Liquid, <sup>32</sup> P	82	81	130	812
Liquid, <sup>51</sup> Cr	24	21	10	25
Solid, <sup>3</sup> H	55	49	151	267
Solid, <sup>14</sup> C	95	85	451	803
Solid, <sup>125</sup> I	83	75	344	625
Solid, <sup>32</sup> P	93	87	432	841
Solid, <sup>51</sup> Cr	48	24	115	64

(compare D9 with F3, which contains about the same activity). Even in this extreme situation, the cumulative crosstalk from the surrounding wells is only about 1% of the average activity in those wells. Thus, although crosstalk with high-energy radionuclides is measurable, one can obtain quantitative results with a dynamic range of 100 or more. These results are obtained with the reporting of only raw, uncorrected data from the instrument.

Glow-type luminescence can be quantitated with the SPC capability of TopCount™. We have quantitated alkaline phosphatase, β-galactosidase, peroxidase, xanthine oxidase, and firefly luciferase in TopCount™ (data not shown). The average individual well crosstalk measured in a black polystyrene microplate (MicroFLUOR®) with xanthine oxidase luminescence was 0.007% (Fig. 3C). The worst individual well crosstalk that we observe with black plates is <0.03% (e.g., Fig. 3C). This level of crosstalk is insignificant, even when low-activity wells are proximal to wells containing about 100 times as much activity (right side of Fig. 3C). SPC crosstalk in white microplates is about ten times greater than in black plates.

## CONCLUSIONS

TopCount™ was designed to be a flexible system for microplate analysis. A design fundamentally different from that of conventional scintillation counters was chosen to achieve efficient counting of the closely spaced samples in microplates without crosstalk. This design has achieved excellent liquid scintillation, solid scintillation and luminescence counting performance for a variety of sample formats. Very low or non-existent crosstalk ensures a dynamic bioassay range of at least 100, even in the most extreme situations with low-activity samples surrounded by high-activity samples.

## REFERENCES

- Baret, A., Fert, V. and Aumaille, J. 1990 Application of long-term enhanced xanthine oxidase-induced luminescence in solid-phase immunoassays. *Analytical Biochemistry* 187: 20–26.
- Beckman Instruments 1989 *ReadyFilter Disks: Common Questions and Answers*. Beckman Instruments Publication CS 1/002.
- Jones, D. K., Tomisek, J. D., Park, E. and Young, H. M. 1986 Reverse sum quench measurement using a liquid scintillation counter. U.S. Patent No. 4,633,088.
- Neumann, K., Englert, D. and Roessler, N. 1993 Biological applications of microplate scintillation counting. This volume.